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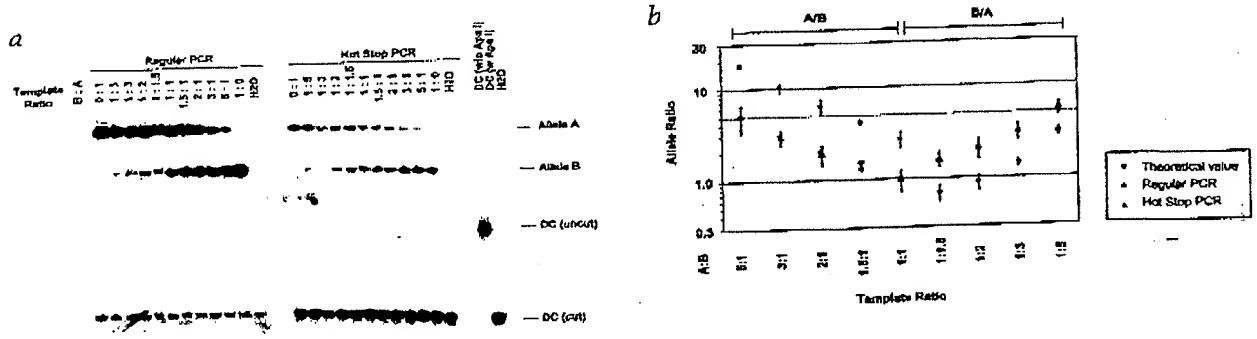

Fig. 2 Comparison of regular PCR and hot-stop PCR for quantitation of allele ratios. *a*, Representative result at 35 cycles of PCR, comparing regular PCR with hot-stop PCR at varying ratios of *IGF2* template, with primers P3 and P4 as described[3]. A allele, 294 bp, undigested by *ApaI*; B allele, 229 bp, digested by *ApaI*; DC, internal digestion control for this experiment (179 bp undigested, 126 bp digested). *b*, Linearity and accuracy of hot-stop PCR compared with regular PCR. Three independent experiments were done and the averages of allele ratios from regular PCR and hot-stop PCR are indicated by green circles and blue triangles, respectively, and standard deviations are indicated by lines. Red squares represent the theoretical values. *c*, Representative results of analysis of *IGF2* imprinting by hot-stop PCR of normal and tumour samples. Normal tissue was from a fetal kidney that showed complete imprinting of *IGF2*. Tumours showed partial or complete LOI. LOI in the cancer represented by the fourth panel is evident despite a 2:1 genomic DNA allele ratio due to aneuploidy. G, + and – indicate that the PCR templates were from genomic DNA, cDNA (RT+) or cDNA (RT–), respectively.

PCR (Fig. 2*a*,*b*). In contrast, using hot-stop PCR, the A:B ratio precisely reflected the theoretical ratio (Fig. 2*a*,*b*). As expected, the allele ratio measured by hot-stop PCR was independent of PCR cycle number, amount of template or PCR amplification efficiency, whereas regular PCR was sensitive to these variables.

We also analysed the imprinting status of *IGF2* in normal and tumour cells. Normal imprinting, LOI and partial LOI were observed and quantified. The assay was also sensitive enough to quantify genomic allele ratio in aneuploid cells, allowing correction of allele-specific expression ratio for the allele-specific genomic template ratio (Fig. 2*c*). These results do not contradict previous studies examining ratios of allele-specific expression, provided that those experiments were performed with careful attention to limiting cycle number and avoiding high template concentration, but they do illustrate the possibility of errors introduced by regular PCR. We expect that hot-stop PCR will allow for more consistent and reliable measurement of small differences in these ratios, an important issue for genes that show subtle tissue-specific changes in genomic imprinting[1–3].

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The *NOTCH4* locus is associated with susceptibility to schizophrenia

Linkage disequilibrium mapping of the MHC region in 80 British parent-offspring trios showed that *NOTCH4* was highly associated with schizophrenia. The A→G substitution in the promoter region and the (CTG)n repeat in exon 1 of *NOTCH4* may be candidate sites conferring susceptibility to schizophrenia.

Linkage studies indicate that chromosome 6p may contain a susceptibility locus for schizophrenia[1–4], although the evidence is not strong enough to draw firm conclusions. It may be that a gene of moderate or small effect rather than one of major effect is involved. Because the human major histocompatibility complex (MHC) region is mapped to 6p21.3 and its complete genomic DNA sequence is now available, we conducted the transmission disequilibrium test[5] (TDT) with densely spaced DNA markers to search for a schizophrenia susceptibility gene within the MHC region.

We examined 80 parent-offspring trios of British descent, consisting of fathers,

Table 1 • LD analysis of the class II and class III regions of the MHC in schizophrenia

Locus	Accession	Marker	Heterozygosity	*P* value[a]
AC004180	AC004180	(GT)n	0.85	0.145
HSY14768	Y14768	(CA)n	0.82	0.16
DJ201G24	AF129756	(GT)n	0.82	0.311
HSA012008	AJ012008	(CA)n	0.67	0.463
HSMIIC3W36A	U89337	(TTTC)n	0.73	0.5
HSMHC3A5	U89335	(TAA)n[b]	0.74	0.00017
		SNP1[c]	0.16	0.267
		SNP2[d]	0.26	0.002
		(CTG)n[e]	0.79	0.000036
		(TTAT)n[f]	0.51	0.393
HS1077I5	AL034394	(CT)n	0.66	0.356
HS172K2	Z84814	(GT)n	0.75	0.5
HSDV19	Z84490	(CA)n	0.73	0.372
HSEVMHC	X87344	(TAAA)n	0.59	0.016
HSO14	Z84497	(GT)n	0.76	0.5
HS1033B10	AL031228	(CCTT)n	0.53	0.5
HSICK721Q	AL021366	(GT)n	0.83	0.108

[a]The TDTLIKE program for the McNemar test was applied to analyse genotyping data[11]. The significance level of a *P* value was set at 0.003 as 17 markers were tested. [b]Frequencies of 8 transmitted alleles were 1, 53, 22, 22, 5, 7, 5 and 4, and of 8 non-transmitted alleles, 1, 21, 35, 32, 7, 13, 4 and 6. [c]The T→C substitution creates a *MspI* site. Frequencies of the 2 transmitted alleles were 30 and 22. [d]The A→G substitution also creates a *MspI* site. Frequencies of the 2 transmitted alleles were 27 and 55. [e]Frequencies of 7 transmitted alleles were 1, 13, 23, 63, 8, 17 and 1, and of 7 non-transmitted alleles, 1, 18, 47, 23, 12, 22 and 3. [f]Frequencies of 6 transmitted alleles were 5, 0, 31, 20, 11 and 2, and of 6 non-transmitted alleles, 9, 3, 24, 23, 9 and 1. Only 68 of 80 parent-offspring trios were available for analysis of the (TTAT)n repeat, as it is unstable in some families.

mothers and affected offspring with schizophrenia. We analysed 13 loci, which together span approximately 1.8 Mb of DNA and cover the class II and class III regions of the MHC. The order of these 13 loci is as follows: 6pter–AC004180–HSY14768–DJ201G24–HSA012008–HSMIIC3W36A–HSMHC3A5–HS1077I5–HS172K2–HSDV19–HSEVMHC–HSO14–HS1033B10–HSICK721Q–6cen. The TDT showed that the HSMHC3A5 locus, lying near the junction of the class II and III regions, is significantly associated with schizophrenia (Table 1). The HSMHC3A5 locus contains the gene *NOTCH4*, spanning approximately 56.8 kb of DNA, inclusive of a putative promoter region and 30 exons[6]. We performed a further study using DNA marker haplotype systems consisting of three microsatellites and two SNPs within the HSMHC3A5 locus. Of the 5 markers, 3 are present in the 5′ flanking region of *NOTCH4*, 1 in exon 1 and 1 in intron 17. Their order is as follows: 5′–(TAA)n–SNP1–SNP2–(CTG)n–(TTAT)n–3′. Of seven DNA marker haplotypes, the SNP2–(CTG)n haplotype showed the strongest association with schizophrenia (P=0.0000078), and the SNP1–SNP2–(CTG)n haplotype showed the second strongest association (P=0.000011; Table 2). To assess the effect of segregation distortion[7], we also analysed 48 unaffected offspring using these five markers. The TDT did not show significant distortion at these marker loci. Our results suggest that the susceptibility gene may be *NOTCH4* or a nearby locus.

The significant level of linkage disequilibrium (LD) extended over the (TAA)n repeat, but the (CT)n repeat in the testis-specific basic protein gene, adjacent to the 5′ end of *NOTCH4* and present in the HS1077I5 locus, was not associated with schizophrenia. Moreover, four known genes (*PBX2*, *AGPAT1*, *AGER* and *TNXA*) and some ESTs encoding unknown proteins lie between *NOTCH4* and the HSMHC3W36A locus. Associations between these genes and schizophrenia cannot be ruled out, although the significant LD level did not extend over the (TTAT)n repeat because it was less informative than the (CTG)n and (TAA)n repeats.

The (CTG)n repeat in exon 1 of *NOTCH4* encodes leucine in the signal peptide domain. We have typed seven alleles using the (CTG)n repeat: $(CTG)_5$, $(CTG)_6$, $(CTG)_9$, $(CTG)_{10}$, $(CTG)_{11}$, $(CTG)_{12}$ and $(CTG)_{13}$. An excess of the $(CTG)_{10}$ allele was transmitted to affected offspring by their parents. Possibly, the $(CTG)_{10}$ allele itself may confer a susceptibility to schizophrenia. The SNP2, an A→G substitution in the promoter region, should also be considered as a candidate site.

Table 2 • Haplotype analysis for association of *NOTCH4* variation with schizophrenia

Haplotype	*P* value
(TAA)n–SNP1	0.0002
SNP1–SNP2	0.0162
SNP2–(CTG)n	0.0000078
(CTG)n–(TTAT)n	0.189
(TAA)n–SNP1–SNP2	0.000028
SNP1–SNP2–(CTG)n	0.000011
SNP2–(CTG)n–(TTAT)n	0.064

The (TAA)n repeat is 8.8 kb away from SNP1; SNP1 is present at base −1,725 of the 5′ flanking region of *NOTCH4* and SNP2 at base −25; and the (CTG)n repeat is 40 bp away from SNP2 and 12.3 kb away from the (TTAT)n repeat.

Little is known about the functions of *NOTCH4* in humans, but animal studies demonstrate that the NOTCH family of proteins has a role in determining cell fate[8]. *NOTCH* was originally characterized as a *Drosophila melanogaster* neurogenic gene required for the correct segregation of epidermal cells from neuronal cell precursors during embryogenesis[9]. *In situ* hybridization in mice showed that although *Notch4* is highly expressed in endothelial cells, its transcripts are also detected in the developing nervous system[10]. These findings raise the possibility that *NOTCH4* may be involved in the development of neurogenic diseases.

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